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(54) Title: IMMUNOSTIMULANTS (57) Abstract A polycationic carbohydrate such as chitosan, or a pharmaceutically acceptable derivative thereof, are used as immunostimulants. Vaccine compositions containing these polycationic carbohydrates, in particular in particles such as microparticles or liposomes are also described and claimed. Methods of treatment and the use of the polycationic carbohydrates as immunostimulants in the production of vaccines are further aspects described and claimed.		

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Immunostimulants

The present invention relates to compounds which are polycationic carbohydrates, for use as immunostimulants and to vaccines containing these. The invention further comprises methods of treating individuals using the pharmaceutical compositions containing the compounds as well as methods of preparing the compositions.

A prime objective in the field of vaccination is the development of a non-parenteral immunisation regimen, which facilitates induction of comparable levels of systemic immunity to that elicited by conventional sub-cutaneous and intra-muscular injections.

The nasopharyngeal passages and pulmonary regions of the respiratory tract represent potential targets for the systemic delivery of peptidergic drugs and vaccines. The relative ease with which therapeutic agents can be inhaled, or introduced into the nose, make these modes of immunisation attractive in terms of probable patient compliance. Furthermore, respiratory mucosae offer certain morphological, physiological and immunological advantages over other non-parenteral sites in terms of immunisation, particularly against pathogenic entities which affect or utilise mucosal surfaces as portals of entry. This is because effective vaccination against these pathogens normally requires mucosae to be adequately protected with locally produced antibodies of the secretory IgA (sIgA) isotype. Whilst mucosal surfaces are usually poorly protected with IgA following parenteral administration of vaccines, it is now apparent that successful delivery of antigenic material to immunoresponsive elements in mucosa-associated lymphoid tissue (MALT) can result in vigorous stimulation of the mucosal arm of the immune system. By means of the common mucosal immune system (CMIS) it is feasible that several anatomically disparate mucosal surfaces could be protected through mucosal

administration of a vaccine at a single site. Mucosal vaccination offers the added advantage that some degree of systemic immunity can be induced in concert with local responses due to translocation of antigenic material from sub-epithelial compartments to systemic immunoresponsive tissues such as the spleen.

Despite the logistical and immunological factors which favour non-parenteral immunisation, simple mucosal application of antigenic proteins, for example in the gastrointestinal or respiratory tracts, is usually ineffectual in terms of vaccination. Enzymatic or chemical destruction, combined with poor absorption into sub-epithelial compartments dictate that mucosally administered vaccines usually require some form of adjuvant or delivery vehicle. One approach is to encapsulate antigenic material within microparticulate polymeric carriers, such as poly-DL-lactide (PLA) microspheres (Vaccine 1994, 12, 5-11). Such procedures serve to protect labile vaccines from lumenal degradation and enhance absorption into mucosal and systemic compartments (J.H. Eldridge et al., Seminars in Hematology, (1993), 30, 16-25). There is good evidence that microencapsulation may also adjuvantise by converting soluble antigenic molecules into particulate species, thus promoting vaccine uptake into antigen presenting cells (APC) (Y. Tabata et al., Adv. Polym. Sci. (1990), 94, 107-141, L. Vidard et al., J. Immunol. (1996), 156, 2809-2818, N. Van Rooijen, Immunol. Today (1990) 11, 436-439) or microfold cells (M-cells) in lymphoid follicles (R.I. Walker et al., Vaccine, 12, 387, 1994, D.T. O'Hagan et al., Vaccine, 1989, 7, 421-424, P.G. Jenkins et al., J. Drug Targeting, 1995, 3, 79-81).

Although comparatively under-investigated, the intra-nasal (i.n.) route is an attractive one for the mucosal delivery of vaccinal entities. The nasal epithelium is accessible and is less exclusive to high molecular weight molecules.

The thickness of the mucus blanket covering respiratory epithelium is relatively thin compared to that of other mucosae, for example the gut where it is in the region of 500 times thicker. Substantially reduced concentrations of proteolytic enzymes and extremes of pH exist in the respiratory tract compared with the gastrointestinal tract.

Furthermore, it is now delineated that nasal associated lymphoid tissues (NALT) have a lymphoepithelium which, like that in the intestinal mucosa, contain M-cells for selective antigen uptake (P. Brandenburg, Immunology of the Lung and Upper Respiratory Tract, (ed. Bienenstock J.) McGraw-Hill, New York, 1984, 28-95). Hence NALT plays an analogous role to other MALT, such as the gut associated lymphoid tissues (GALT), in terms of antigen surveillance and induction of mucosal and systemic immunological responses.

Polycationic carbohydrates such as chitosan as well as various derivatives or composites of these proteins have been used previously in the pharmaceutical compositions as absorption enhancers to increase delivery of an active pharmaceutical agent across a barrier such as a mucosal membrane. The applicants have found however, that when these compounds are used in the context of vaccine compositions, they act as adjuvants, producing an increase in the immune response to the antigenic agent being delivered. The increase in the response levels persists for considerably longer than would be expected as a result only of absorption enhancing effects.

Thus according to the present invention there is provided a polycationic carbohydrate or a pharmaceutically acceptable derivative thereof, for use as an immunostimulant.

As used herein, the term "immunostimulant" refers to an adjuvant which stimulates the immune system of a host animal to which it is administered and thereby increases the protective effect

produced by a protective antigen administered to that animal, as compared to the effect which would be produced by administration of the protective antigen alone.

5 The expression "polycationic carbohydrate" includes polymeric compounds with repeat units of general formula $C_x(H_2O)_y$. They will include multiple cationic functions or group such as amino or amido groups which are cationic in nature in that they will readily form cations such as quaternary ammonium compounds.

10

Particular examples of polycationic carbohydrates include immunostimulant chitin derivatives such as chitosans or chemically modified forms such as soluble forms thereof; cationic polypeptides; cationic polyamino acids; and quaternary
15 ammonium compounds; or mixtures thereof.

20

Examples of water-soluble derivatives of polycationic carbohydrates in particular, are water-soluble chitin derivatives such as an alkylated chitosan derivatives and salts thereof.

25

Examples of these chemicals include trimethyl chitosan chloride, carboxymethyl chitosan, N-carboxymethyl chitosan, and polyethylene glycol chitosan. N-trimethyl chitosan chloride (TMC) has been referred (Kotze, A. F. et al. Pharm Res. (1997) 14:1197-1202) as a potential absorption enhancer of peptide therapies across mucosal membranes. In contrast to chitosan, TMC is water soluble in all gastrointestinal pH environments. Further, it retains the ability to temporarily open tight
30 junctions. An important parameter appears to be the degree of trimethylation.

35

Other examples of chitosan derivatives which might be used in the context of the invention include chitosan chloride and chitosanglutamate.

Examples of biologically active agents which are capable of generating a protective immune response in an animal, particularly a mammal, and which may benefit from combined use with the immunostimulants of the invention are well known in the art. They include antigenic polypeptides as well as nucleic acid sequences which may encode these polypeptides and which are known as "naked DNA" vaccines. Live vaccines such as DNA or RNA viral vaccines may also be used in conjunction with the immunostimulant of the invention.

As used herein the expression "polypeptide" encompasses proteins or epitopic fragments thereof.

Suitable polypeptides are sub-unit vaccines, such as tetanus toxoid, diphtheria toxoid and *Bacillus anthracis* protective antigen (PA).

A particular example of a biologically active agent is one which is capable of generating a protective immune response against *Yersinia pestis*. The agent is suitably a sub-unit vaccine, for example as described in WO 96/28551. The vaccine described and claimed there comprises a combination of the V antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these, and the F1 antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these.

As used herein, the term "fragment" refers to a portion of the basic sequence which includes at least one antigenic determinant. These may be deletion mutants. One or more epitopic region of the sequence may be joined together.

The expression "variant" refers to sequences of nucleic acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced

with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% homologous, preferably at least 75% homologous, and more preferably at least 90% homologous to the base sequence. Homology in this instance can be judged for example using the algorithm of Lipman-Pearson, with Ktuple:2, gap penalty:4, Gap Length Penalty:12, standard PAM scoring matrix (Lipman, D.J. and Pearson, W.R., Rapid and Sensitive Protein Similarity Searches, *Science*, 1985, vol. 227, 1435-1441).

Previously, we have demonstrated that the intranasal (IN) route is a highly effective, non-invasive alternative, to the parenteral administration of recombinant subunit vaccines (F1 and V derived from the causative organism of plague: *Yersinia pestis*) (Eyles, J. E. et al. Vaccine (1998) 16:698-707; J. Drug Targeting Nasal delivery of vaccines) 1996 Alpar & Almeida). Fraction 1 (F1) subunit (molecular mass 15.5 kDa) is derived from the capsule that surrounds the bacteria. In solution, because of its hydrophobic nature, F1 tends to aggregate into multimeric complexes of high (>200,000 kDa) molecular weight (Voronisov, E. D. et al. Biomed. Sci. (1990) 1:391-396 and Miller, J. et al. FEMS Immun. Med. Micro. (1998) 21:213-231). The V antigen (molecular mass 37 kDa) is a protein secreted by the bacterium at 37°C (Leary, S. E. C. et al. Infect. Immun. (1995) 63:2854-2858). V is a virulence factor which may exert local anti-inflammatory effects through modulation of tissue cytokine levels (Nakajima, R. and R. R. Brubaker Infect. Immun. (1993) 61:23-31). Both F1 and V are protective, and there is a documented 'synergistic effect' in combination (Williamson, E. D. et al. Vaccine (1996) 14:1613-1619). Traditionally killed whole cell vaccines for plague have an unsatisfactory incidence of transient local and systemic side effects, but more

importantly, may fail to protect individuals from the pneumonic form of the disease, which is transmissible via airborne droplets (Perry, R. D. and J. D. Fetherston. Clin. Microbiol. Rev. (1997) 10:35-66). Intramuscular injection (Williamson, E. D. et al. Vaccine (1997) 15:1079-1084) of combined F1 and V, or intranasal administration of microspheres co-encapsulating F1 and V (Eyles, J. E. et al. Vaccine (1998) 16:698-707), can protect experimental animals against a lethal inhalational challenge with *Y. pestis*.

10

Following these experiments it was seen that systemic immunity, in the form of high serum IgG titres to F1 and V, was critical for protection. In the current work, the applicants have nasally instilled F1 (5 µg) and V (1 µg) in the presence and absence of a variety of chemicals including three different TMC derivatives (with increasing degrees of quaternization: 20, 40 and 60%), and compared humoral immune response engendered by these treatments to those evoked by co-administration of chitosan itself. The results indicate that the higher the degree of derivitisation, the greater the increase in the immune response. Thus TMC 60 is the most preferred form.

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In a further aspect, the invention provides a method of protecting an animal against a pathogen, said method comprising administering to said animal, a protective agent which is able to stimulate the animal's immune system to produce a response which is protective against said pathogen, and an immunostimulant comprising a polycationic carbohydrate.

30 Suitable animals are mammals including humans.

The adjuvant or immunostimulant may be administered simultaneously with said protective agent, suitably in a combined formulation. Alternatively, it may be administered separately. The selection of particular administration conditions will vary depending upon factors such as the nature

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of the protective agent, the condition being treated, the age and size of the animal etc.

Preferably however the immunostimulant of the invention will be administered to the animal in a single pharmaceutical composition with the protective agent.

Suitably the composition will further comprise a pharmaceutically acceptable carrier or diluent. These may be solid or liquid carriers as are known in the art. Carriers or diluents may vary depending upon the particular nature of the protective agent and the particular polycationic carbohydrate used. They may comprise pharmaceutically acceptable solvents such as water in which the protective agent and the polycationic carbohydrate are dissolved. This type of formulation is particularly suitable when the protective agent is itself water-soluble.

Compositions in the form of solutions of this type suitably contain from 0.1 to 30% w/v and preferably from 1 to 20% w/v of polycationic carbohydrate, depending upon its solubility.

The composition is suitably adapted for parenteral administration such as intramuscular (i.m.) administration, or it may be suitable for administration to mucosal surfaces of the animal.

Administration to mucosal surfaces may be effected by oral application, by pulmonary application, for example by intra-tracheal administration, or particularly by intra-nasal application. In particular, the compositions of the invention are administered by the intra-nasal route.

For many applications however, it has been found preferable that the protective agent and the polycationic carbohydrate immunostimulant is microencapsulated in a polymeric material and

thus the carrier is a particulate carrier such as a microsphere or microparticle (also known as a microcapsule), nanocapsule or liposome.

5 In a preferred embodiment, a polycationic carbohydrate which is able to act as an immunostimulant is provided in the form of a pharmaceutical composition, which composition comprises particles comprising

- (i) a biologically active agent which is able to produce an
10 immune response in an animal to which it is administered;
- (ii) a first material capable of forming particles; and
- (iii) said polycationic carbohydrate.

The particles are suitably incorporated into a composition which
15 is adapted for administration to mucosal surfaces. In addition, the particles may suitably be incorporated into a composition which is adapted for administration using a parenteral route such as i.m. injection.

20 Suitably the particles comprise microparticles or microspheres or liposomes.

The first material used in the compositions of the invention is suitable for forming microspheres or liposomes. Liposome
25 production requires the use of lipids and/or surfactant type molecules as is understood in the art.

Preferably however, the composition of the invention comprises a microsphere. In this case the first material comprises a
30 polymer. It may be a low, medium or high molecular weight polymer. Examples of low molecular weight polymers are polymers which have a molecular weight of between 0.1 and 10kDa, more preferably between 1 and 5 kDa and typically about 2-3kDa.

35 The use of high molecular weight polymers in the encapsulation of a tetanus vaccine for intramuscular administration has been

described (Vaccine 1994, 12, 4, 299-306). A formulation of microencapsulated ricin toxoid vaccine which is applied intranasally has also been described (Vaccine 1994, 14, 11 1031). However, in that case, high molecular weight polymer
5 microparticles (94kDa) were less effective than those prepared from a copolymer of lower molecular weight (72kDa).

The polymeric material used as the first material in the composition of the present invention suitably has a high
10 molecular weight in excess of 94kDa, for example of 100kDa or more.

A particularly suitable polymeric first material for use in the compositions of the invention comprises poly-(L-lactide) or PLA
15 but other high molecular weight polymeric material such as poly(lactic/glycolic acid) PGLA, polycyanacrylates, polyanhydrides or polycaprotactones as are known in the art may be employed.

Suitably the polycationic carbohydrate is present in the composition in an amount of from 0.1% to 10%w/w. Particular examples of suitable polycationic carbohydrates for use in the above described particle compositions include chitin derivatives such as chitosans; cationic polypeptides; cationic polyamino
25 acids; and quaternary ammonium compounds; or mixtures thereof.

The compositions may optionally further comprise agents which stabilise emulsions such as polyvinylalcohol or methyl
cellulose.

30 They will suitably be of an average size of from 0.1 μ m to 10 μ m in diameter.

Optionally, vaccine compositions may further comprise an
35 additional conventional adjuvant in order to enhance the immune response to the biologically active material administered.

Suitable adjuvants include pharmaceutically acceptable adjuvants such as Freund's incomplete adjuvant, aluminium compounds and, preferably adjuvants which are known to up-regulate mucosal responses such as CTB, the non-toxic pentameric B subunit of cholera toxin (CT).

Yet further adjuvants are described and claimed in the applicants copending International application of even date, derived from British Patent Application Nos. 9906694.6 and 9906696.1. In particular these compounds are

- A) a polyamino acid,
- B) a vitamin or vitamin derivative,
- C) cationic pluronics,
- D) a clathrate,
- E) a complexing agent,
- F) cetrimides;
- G) an S-layer protein; or
- H) methyl-glucamine.

As used herein, the expression "cationic pluronics" includes both pluronics which include cations, as well as those which have been treated such that they are bound to cationic moieties (cationised pluronics).

Suitable biological agents (i) include drugs and therapeutic molecules such as vaccines, antivirals, antibiotics, antifungals, antiparasitics as well as oligonucleotides used in therapies and vaccines.

However in a preferred embodiment, the biologically active agent is an agent that is capable of generating an immune response in an animal to which it is administered and most preferably a protective immune response. Thus the compositions are suitably used as vaccines including those which rely on oligonucleotides or other nucleic acid sequences. In this case, the immunostimulant properties of the compounds A-H are used.

Suitably the said adjuvant chemical is soluble in water.
Suitably the composition is suitable for non-parenteral
administration for example to mucosal surfaces or for topical
5 application to the skin. Particularly preferred compositions
are suitable for administration to mucosal surfaces.

Alternatively, the composition is suitable for parenteral
administration for example by intramuscular (i.m.)
10 administration.

Administration to mucosal surfaces may be effected by oral
application, by pulmonary application, for example by intra-
tracheal administration, or particularly by intra-nasal
15 application. In particular, the compositions of the invention
are administered by the intra-nasal route.

Examples of adjuvant chemicals in category (A) above include
polyamino acids such poly-ornithine, for example of molecular
20 weight from 5 to 150kDa.

Particular examples of adjuvant chemicals in category (B) above
are vitamins or vitamin derivatives such as vitamin E or
derivatives for example vitamin E TPGS (d-alpha tocopheryl
25 polyethylene glycol 1000 succinate).

Particular cationic pluronics in category (C) above, are block
copolymers or surfactants which are positively charged, in
particular with NH_2^+ groups. These are available commercially
30 for example from ICI Ltd (UK) sold under the trade names P101
and P121.

Examples of clathrates in category (D) above include in
particular cyclodextrins and their derivatives such as dimethyl
35 β cyclodextrin.

Suitable complexing agents in category (E) above are bile salts, in particular those which form complexes with fatty acids such as deoxycholic acid.

- 5 Examples of cetrimides in category (F) are quaternary ammonium compounds used as preservatives.

In particular, the compositions of the invention may further comprise a cationic pluronic as described above. These may be
10 included either within the particles, or as a component of the dosing mixture or both. In a particularly preferred embodiment, the cationic pluronic is formed into microparticles and in particular nanospheres, which are then coated with the polycationic carbohydrate such as chitosan. Biologically active
15 material may then be absorbed onto the coated particles.

They may also comprise other known composition components such as colouring agents and preservatives and in particular cetrimide. These are suitably present in amounts of from 0.1 to
20 0.7%w/v.

In a particular embodiment, the microspheres or liposomes used in the compositions may further comprise a S-layer proteins, in particular, S-layer proteins derived from a bacteria against
25 which the biologically active agent produces a protective immune response. These may be distributed throughout the microspheres or liposomes but are preferably coated on the surface. It has been shown (Sleytr et al., Crystalline bacterial cell surface proteins. Biotechnology Intelligence Unit, 1996, R.G. Landes
30 Company and Academic Press Inc.) that the stability of liposomes can be increased by such coatings. S-layer proteins are found on the surface of most bacteria and form a regular two dimensional array known as an S-layer. Isolated S-layer proteins are able to form entropy driven monomolecular arrays in
35 suspension, and on the surface of structures such as liposomes.

The above-described particle composition is particularly suitable for intranasal application. They may comprise particles such as microparticles per se which are optionally preserved, for example by lyophilisation, or the microparticles
5 may be combined with a pharmaceutically acceptable carrier or excipient. Examples of suitable carriers include solid or liquid carriers as is understood in the art.

The invention further provides a method of producing a
10 pharmaceutical therapeutic vaccine, which method comprises encapsulating a biologically active agent as described above in a first polymeric material which has a high molecular weight and in particular a molecular weight of 100kDa or more, in the presence of a polycationic carbohydrate such as chitosan. The
15 polycationic carbohydrate may be incorporated within the microparticle, or at the surface, or preferably is distributed throughout the microparticle including at the surface.

In some cases, particularly where the polycationic carbohydrate
20 is insoluble, it may preferably be adsorbed onto the surface of a pre-formed particle. It is possible also to load biologically active agent (such as a protective antigen) onto the thus formed particles.

25 Methods of forming liposomes are well known in the art. They include dispersion of dehydrated lipid films into aqueous media, emulsion techniques and lyophilisation methods as are well known in the art.

30 Microparticles of the invention are suitably prepared using a double emulsion solvent evaporation method. Briefly, the biologically active agent, suitably in a lyophilised state, is suspended in an aqueous solution of the first polymer such as polyvinyl alcohol (PVA) and the polycationic carbohydrate such
35 as chitosan. A solution of the high molecular weight polymer in an organic solvent such as dichloromethane, is added with

vigorous mixing. The resultant emulsion is then dropped into a secondary aqueous phase, optionally containing polycationic carbohydrate, with vigorous stirring. After addition, the organic solvent is allowed to evaporate off and the resultant
5 microspheres separated.

Preferred biologically active agents and first materials and polycationic carbohydrates are as described above.

10 The compositions will suitably comprise an appropriate dosage unit of the active agent. This will vary depending upon the nature of the active agent being employed, the nature of the patient, the condition being treated and other clinical factors. In general however, the composition of the invention will
15 comprise approximately 2 to 10 wt% of active ingredient.

In microparticle containing compositions of the invention the amount of first material, in particular the high molecular weight polymer, in the composition will be of the order of 70 to
20 99wt% of the composition, and suitably from 90 to 99wt% of the polymer components will be the first polymer. The amount of polycationic carbohydrate material, such as chitosan or a mixture of chitosan with other positively charged molecules, will be of the order of 0.1 to 10 wt % of the composition.

25

-In use, a reasonable dosage for nasal administration would be of the order of 0.05g.

Thus, in a further aspect, the invention provides a method of
30 protecting a mammal against infection, which method comprises administration of a vaccine composition as described above to a mucosal surface, in particular a nasal surface, of a mammal.

The applicants have demonstrated that it is possible to protect
35 experimental animals from inhalation challenge with *Y. pestis* through i.n. administration of a combined sub-unit vaccine. The

adjuvantisation of these sub-units is advantageous in enhancing the immune response as is microencapsulation of the sub-units. The high molecular weight polymer utilised in the compositions of the invention appears to be particularly well suited to
5 intra-nasal delivery.

Alternatively, however, the immunostimulants of the invention may be administered as i.m. formulations and long term beneficial effects are still noted.

10

The invention will now be particularly described by way of example with reference to the accompanying drawings in which:

Figure 1 illustrates the serum immune response in mice to
15 nasally delivered microencapsulated and free diptheria toxoid with 10 lf units on day 1 and day 67, where the first column represents results with microsphere without chitosan, the second column represents the results of microspheres with chitosan and the third column shows the results with free diptheria toxoid
20 alone;

Figure 2 illustrates the specific serum antibody responses following a single nasal application of 1µg V and 5µg F1 antigens of *Yersinia pestis* in compositions according to the
25 invention; and

Figure 3 shows the specific serum antibody responses over a period up to 86 days following intramuscular injection of free of microencapsulated BSA in the presence or absence of chitosan;
30

Figure 4 shows the serum immune response to nasally delivered Diptheria toxoid (DT) adsorbed onto nanoparticles whose surface has been modified cationic with chitosan derivatives;

35 Figure 5 shows the results of photon correlation spectroscopy (PCS) on particles produced for use in the invention, showing

that the number mean diameter (d_n) and the volume mean diameter (d_v) were both around 150nm; and

Figure 6 illustrates the immune response to Diptheria toxoid

- 5 (DT) in various formulations applied by i.m. routes, where P101 is a pluronic 101 and P121 is a block co-polymer available from ICI Ltd, UK.

Example 1

10 Microencapsulation of diptheria toxoid

Poly-L-lactide of molecular weight 100kDa (Polysciences Inc.

USA) was used in a modification of the double emulsion solvent evaporation method (Y. Ogawa et al., Chem. Pharm. Bull., 36

(1988) 1095-1103). Briefly, 1.5ml of a 0.75% w/v of chitosan

- 15 solution containing diptheria toxoid was vigorously mixed with 200mg of 100K PLA polymer dissolved in 5ml of HPLC grade

dichloromethane (DCM) using a Silverson homogeniser (Silverson,

UK) for 1 minute. The resultant primary emulsion was added,

- 20 0.5%w/v chitosan and homogenised using a Silverson homogeniser for 5 minutes. This secondary phase was gently stirred

overnight until the dichloromethane had evaporated.

Microspheres were recovered by centrifugation, washed with

double distilled water three times and then lyophilised.

25

Example 2

Immunisation Study

Balb/c female mice (25g, 6-week old) were lightly anaesthetised using an inhaled gaseous mixture of 3% halomethane (RMB Animal

- 30 Health Ltd., UK) in oxygen ($300\text{cm}^3\text{min}^{-1}$) and nitrous oxide ($100\text{cm}^3\text{min}^{-1}$) for intranasal dosing procedures. Groups of mice received one of the following treatments:

(1) Microspheres prepared as described in Example 1 but in the absence of chitosan;

- 35 (2) Microspheres prepared as described in Example 1; and

(3) Free diptheria toxoid solution.

Each were administered in 50µl of PBS using a micropipette. Groups of mice each received 10 lf units on day 1 and day 67 of either microencapsulated or free diptheria toxoid.

5 Serum immune responses were monitored. Tail vein blood samples were taken from all animals on days 14, 28, 95 and 151 of the experiment. Titration of IgG and IgA antibody isotypes in serum samples was achieved using an ELISA. Briefly, individual serum samples were aliquoted to microtitre plates pre-coated with
10 diptheria toxoid. Binding of serum antibody was detected with peroxidase-labelled secondary antibody to mouse IgG (Sigma A4416) or AgA (Sigma A4789). Antibody titre was estimated as the maximum dilution of the serum giving an absorbance reading greater than the maximum optical density (OD) of titrated naïve
15 serum. From this, mean titres \pm standard deviation (SD) were derived per treatment group.

The results are shown in Figure 1. Throughout the 151 day schedule, mice dosed with microencapsulated antigen in the
20 absence of chitosan (Group 1) maintained statistically elevated serum IgG titres to diptheria toxoid in comparison to animals treated with free vaccine (Group 3) (Figure 1). However, the levels of IgG titres to diptheria toxoid in Group 2 animals was consistently higher still indicating that the presence of
25 chitosan in the microparticle enhances the immune response to the toxoid.

Example 3

Use of absorption enhancers to enhance immunological response to
30 intranasally administered subunit vaccines

Five groups of five (n=5) BALB/c mice were intranasally immunised with admixed F1 (5 µg) and V (1 µg). The five treatment groups received the subunits in conjunction with
35 either: 1) Phosphate buffered saline (pH 7.4); 2) 0.2% w/v

chitosan HCL; 3) 0.2% w/v TMC 60; (4) 0.2% w/v TMC 40; (5) 0.2% w/v TMC 20. A further group of animals acted as a control.

Mice were lightly anaesthetised with an inhaled gaseous mixture of 3% (v/v) halothane (RMB Animal Health Ltd., UK) in oxygen (300cm³ min⁻¹) and nitrous oxide (100cm³ min⁻¹) for i.n. dosing procedures. Each mouse received a 15 µl volume of liquid administered with a micropipette. Tail vein blood samples were taken on day 14, and serum was analysed for the presence of anti-V and anti-F1 IgG antibodies using an indirect ELISA protocol (Eyles, J. E. et al. Vaccine (1998) 16:698-707).

The results (Figure 2) indicate that mucosal co-administration of TMC60 or TMC40 augments the humoral response to F1 and V above and beyond that generated by i.n. instillation of F1 and V in phosphate buffered saline or chitosan HCl. TMC20 failed to improve titre to V, although the effect on immunity to F1 was comparable with that of the more substituted chitosan derivatives (TMC40 & 60).

Example 4

Intramuscular administration of immunostimulants.

Microspheres incorporating bovine serum albumin (BSA) were prepared from poly-L-lactic acid of molecular weights 2kD, 50kD or 100kD (or a combination of these) in the presence or absence of chitosans using a double emulsion method (dichloromethane/water). These preparations were administered to mice in a single intramuscular dose in a 50µl volume of sterile PBS ('Day 0' of the study, 15µg BSA equivalent, typically 1mg microspheres). Blood samples were taken from mice at a number of subsequent timepoints and the serum analysed by ELISA for anti-BSA IgG levels.

The results are shown in Figure 3. In this graph, the following key applies:

M= intramuscular;

5 F = free BSA;

E5 = BSA encapsulated into microspheres composed from a 1:1 mass ratio of 2kD:100kD PLA;

LC = low MW chitosan.

10 Surprisingly it was found that free antigen plus microencapsulated BSA in the presence of chitosan performed better in vivo than other dosing administrations containing only one of these components with free antigen, or free antigen administered alone.

15

Example 5

Surface modification using chitosans

500mg of PDLA (124kD) was dissolved in dichloromethane (DCM).

To this solution, an aqueous solution of 2.5%(w/v)

20 polyvinylalcohol (PVA) was added while probe-sonicated. The resultant primary emulsion was added to 20ml of a cooled aqueous solution of 1.5% (w/v) PVA under homogenisation (Silverson SL-2 homogeniser), to form a secondary emulsion. A further 10ml of water was added to the resulting emulsion and the formulation
25 stirred overnight. Particles were washed and collected by centrifugation with distilled water at 3x20 min at 15,000rpm, before finally being freeze-dried.

Four batches of particles were prepared as described above,
30 three of which were surface-modified, either with N-carboxymethyl chitosan (Canada), chitosan chloride or chitosan glutamate (Pronovo, Norway) by adding 10mg of the relevant chitosan to 90mg of particles suspended in 1 ml of water. This mixture was placed in a bath-sonicator for 5 minutes before being shaken for
35 one hour and finally freeze-dried. The fourth batch remained unmodified.

Saline solution (0.5ml) containing 100Lf diphtheria toxoid was added to 10mg of the particles. Resulting suspensions were shaken overnight to enable adsorption of the antigen to the particle surfaces. following incubation, particles were washed by centrifugation with distilled water, as described above. In all cases, the antigen loading was determined to be in the region of 0.5% (w/w), giving an encapsulation efficiency of generally 50-60%.

Particles were fully characterised and mean particle diameters were found to be around 340nm for all batches.

Groups of Balb/c mice (n=5 per group) were dosed intranasally with the four freshly prepared suspensions, with doses equivalent to 5Lf units of DT per mouse, and a dosing volume of 20µl. Animals were bled periodically and ELISAs carried out to determine serum levels of anti-DT specific IgG titres.

The results for blood collected on Day 28 are shown in Figure 4.

Example 6

Immune responses to intramuscularly delivered diphtheria toxoid (DT)

Eight formulations of pluronic/chitosan nanoparticles were prepared, by a simple sonication method. Deacetylated high molecular weight chitosan was obtained from Fluka. Three process variables were : type of Pluronic used (P101, P121 obtained from ICI Limited, UK), volume of pluronic added (75µl or 200µl per 2ml water), and addition or omission of chitosan. Briefly, to 2ml of double-distilled water, a small volume (75µl or 200µl) of the appropriate pluronic liquid (P101 or P121) was added. Mixtures were vortexed for one minute and sonicated for a further one minute. In order to coating the pluronic particles with chitosan, 100µl of a solution of 0.1% w/v high

molecular weight chitosan in 2% w/v glacial acetic acid was added to 100µl of each formulation. Finally, the diphtheria toxoid (DT) was adsorbed to the 200µl of coated and non-coated particles by the addition of 12µl of a solution of DT in water (4450lf units per ml). This preparation is a colloidal dispersion in water with a mean particle diameter of generally between 100-600nm. An example of a typical photon correlation spectroscopy (PCS) printout is shown in Figure 5.

10 Following characterisation of the prepared particulate formulations, groups of four or five female Balb/c mice were given a single dose of 50µl intramuscularly. The total equivocal dose for each animal was 5Lf units of DT. The final concentration of pluronics in the dosing medium was 5% (v/v) and
15 chitosan 0.05% (w/v). Animals were bled periodically and ELISAs carried out to determine serum levels of anti-DT specific IgG titres.

Mean serum anti-DT IgG titres are shown herewith in Figure 6.

20 It is clear from these results, that pluronics produce an enhanced immune response, either alone or in combination with chitosan.

Claims

1. A polycationic carbohydrate or a pharmaceutically acceptable derivative thereof, for use as an immunostimulant.

5

2. A polycationic carbohydrate according to claim 1 which is an immunostimulant comprising a chitin derivative, cationic polypeptide, cationic polyamino acid, a quaternary ammonium compound or a mixture thereof.

10

3. A polycationic carbohydrate according to claim 1 or claim 2 which comprises a chitin derivative.

4. A polycationic carbohydrate according to claim 3 wherein the chitin derivative is chitosan.

15

5. A polycationic carbohydrate according to claim 3 or claim 4 where the polycationic carbohydrate is a water-soluble chitin derivative.

20

6. A polycationic carbohydrate according to claim 5 wherein said water-soluble chitin derivative is an alkylated chitosan derivative or a salt thereof.

25

7. A polycationic carbohydrate according to claim 6 in which the alkylated chitosan is a trimethylchitosan.

8. A pharmaceutical composition comprising a biologically active agent which is capable of generating a protective immune response in an animal, and a polycationic carbohydrate according to any one of claims 1 to 7.

30

9. A pharmaceutical composition according to claim 8 which further comprises a diluent or carrier.

35

10. A pharmaceutical composition according to claim 9 which comprises particles comprising

(i) a biologically active agent which is able to produce an immune response in an animal to which it is administered;

5 (ii) a first material capable of forming particles; and

(iii) a polycationic carbohydrate according to any one of claims 1 to 6.

10 11. A composition according to claim 10 wherein the particle comprises microspheres, microparticles or liposomes.

12. A composition according to claim 10 wherein the particle comprises a microparticle.

15 13. A composition according to any one of claims 10 to 12 wherein the first material is a polymeric material which has a molecular weight of 100kDa or more.

20 14. A composition according to any one of claims 10 to 13 wherein the first material comprises poly-(L-lactide).

15 15. A composition according to any one of claims 10 to 14 wherein the ratio of the first material to the polycationic carbohydrate is from 99:1 to 9:1 w/w.

25 16. A composition according to any one of claims 8 to 15 wherein the biologically active agent is capable of generating a protective immune response against tetanus, diptheria, or *Yersinia pestis*.

30 17. A composition according to claim 16 wherein the biologically active agent comprises a combination of the V antigen of *Y. pestis* or an immunologically active fragment thereof, and the F1 antigen of *Y. pestis* or an immunologically
35 active fragment thereof.

18. A composition according to any one of claims 8 to 17 which is adapted for intranasal application.

19. A composition according to any one of claims 8 to 18 which is adapted for parenteral administration.

20. A composition according to any one of claims 8 to 19 which further comprises an chemical compound selected from

- A) a polyamino acid,
- B) a vitamin or vitamin derivative,
- C) cationic pluronics,
- D) a clathrate,
- E) a complexing agent,
- F) cetrinides;
- G) an S-layer protein; or
- H) methyl-glucamine.

21. A composition according to claim 10 which further comprises a cationic pluronic.

22. A composition according to claim 21 which comprises nanospheres of a cationic pluronic which are surface modified with chitosan.

23. A method for producing a pharmaceutical composition, which method comprises encapsulating a biologically active agent in a first material, in the presence of a polycationic carbohydrate according to any one of claims 1 to 6.

24. A method for producing a pharmaceutical composition which method comprises forming a microsphere, depositing a layer of polycationic carbohydrate according to any one of claims 1 to 6 thereon, and thereafter adsorbing a biologically active agent.

25. A method of protecting an animal against a pathogen, said method comprising administering to said animal, a protective

agent which is able to stimulate the animal's immune system to produce a response which is protective against said pathogen, and an immunostimulant comprising a polycationic carbohydrate.

- 5 26. A method according to claim 25 wherein the protective agent which is able to stimulate the animal's immune system to produce a response which is protective against said pathogen, and an immunostimulant comprising a polycationic carbohydrate is applied parenterally or to a mucosal surface.

10

27. A method according to claim 26 wherein the protective agent and the immunostimulant are applied to a mucosal surface.

15

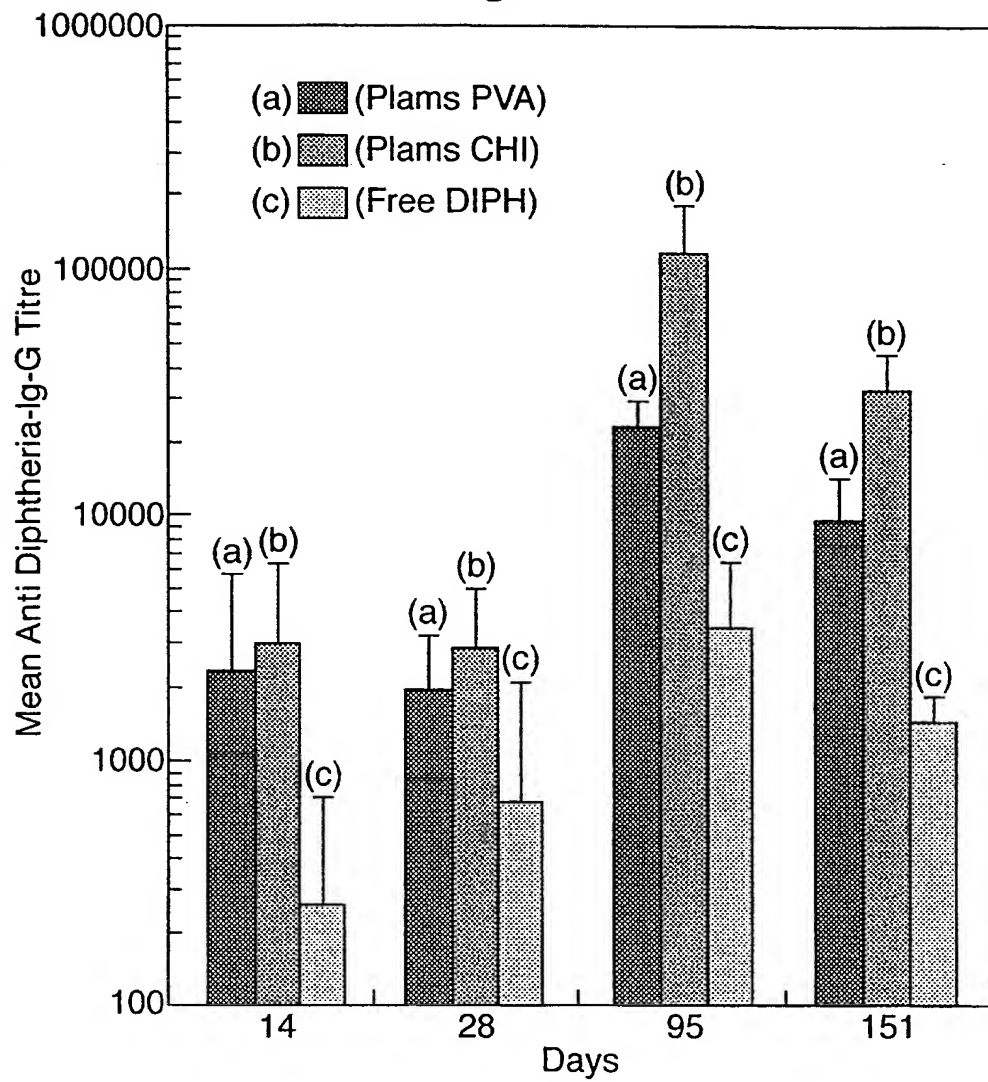
28. A method according to claim 27 wherein said mucosal surface is an intranasal surface.

20

29. The use of a polycationic carbohydrate or a pharmaceutically acceptable derivative thereof as an immunostimulant, in the preparation of a vaccine for use in prophylactic or therapeutic treatment.

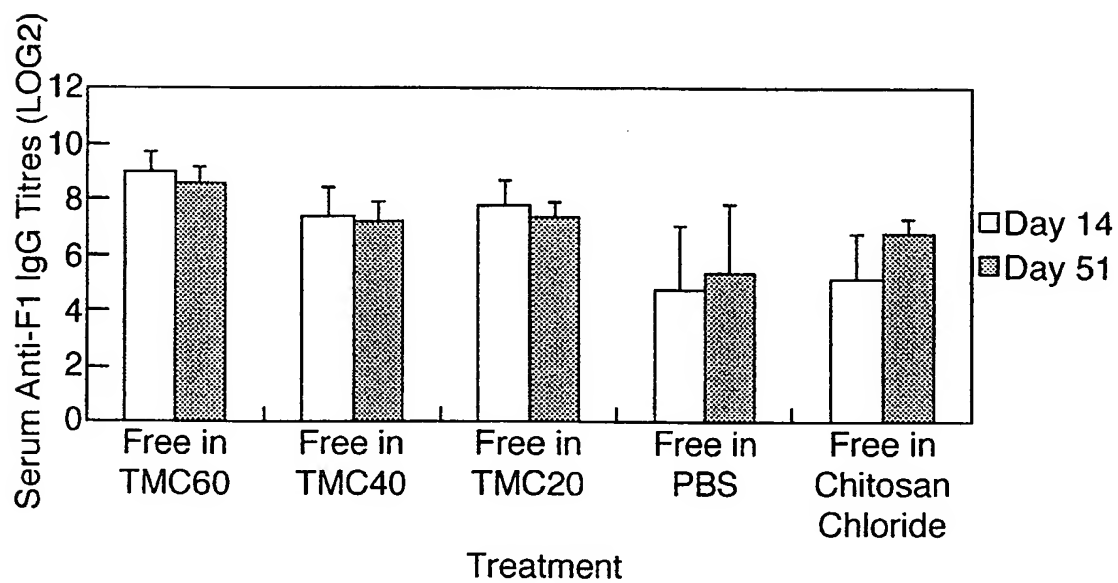
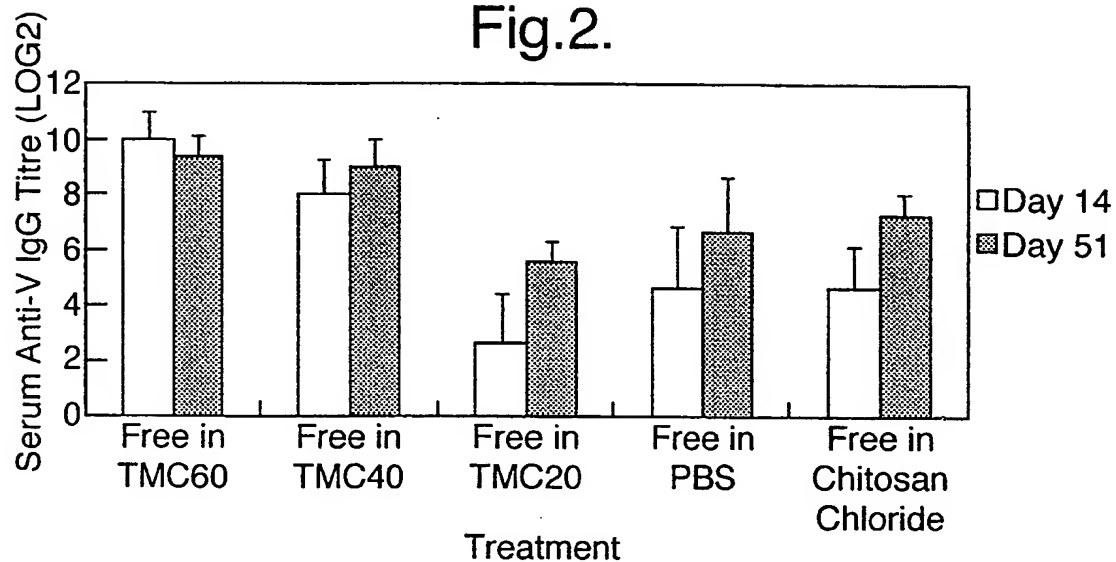
1/6

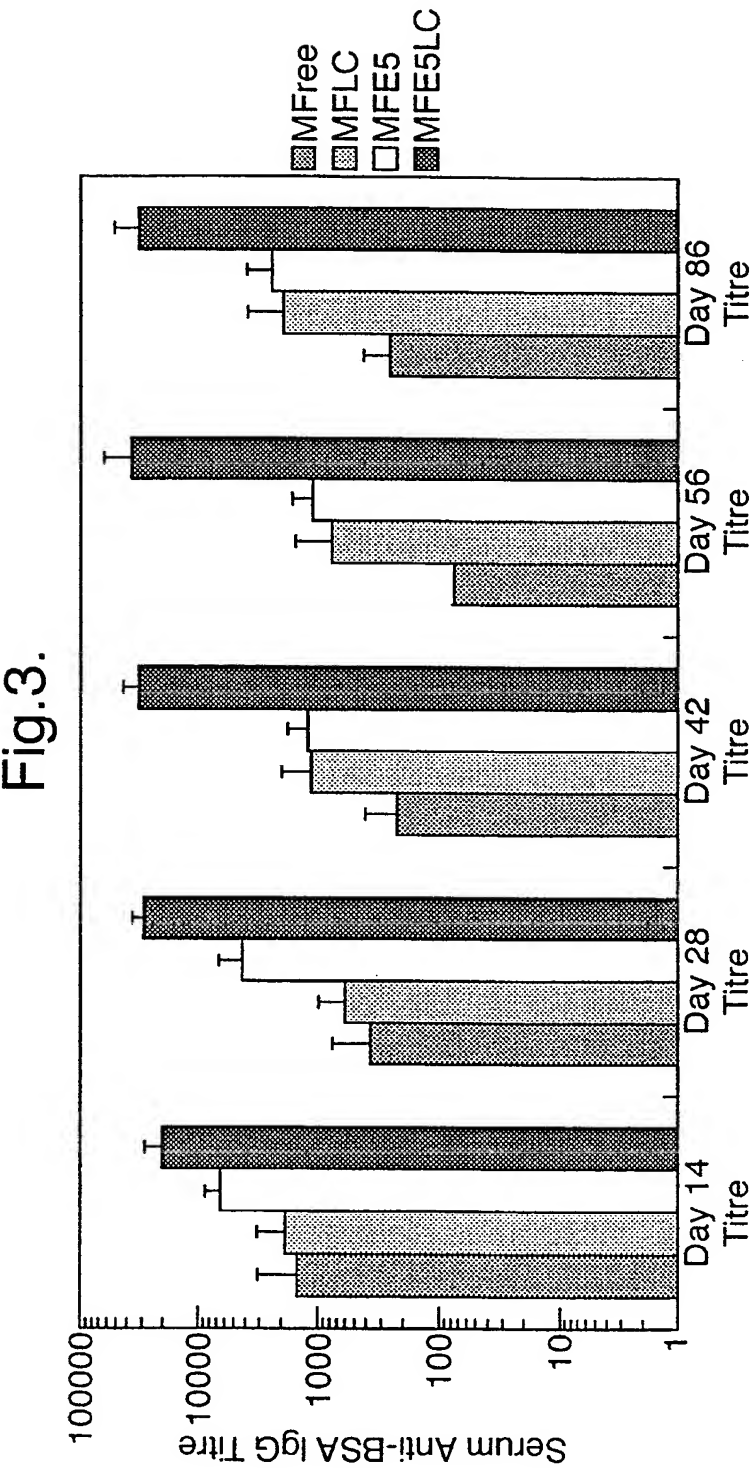
Fig.1.



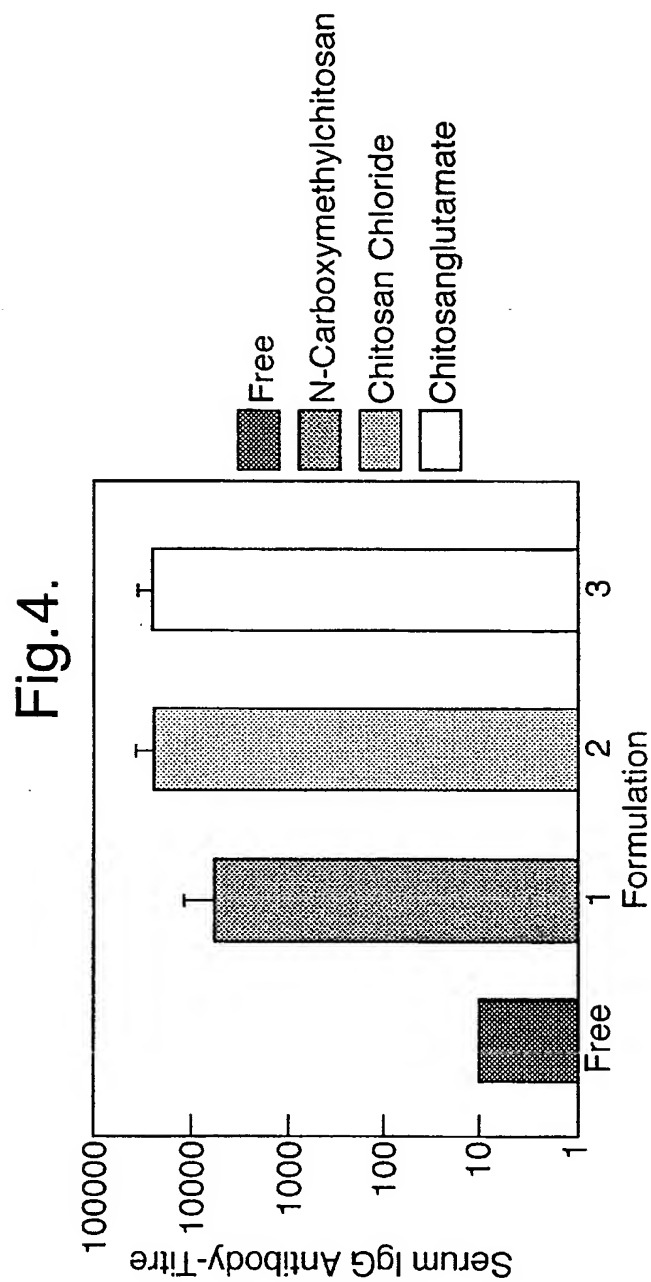
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Fig.2.





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Fig.5.

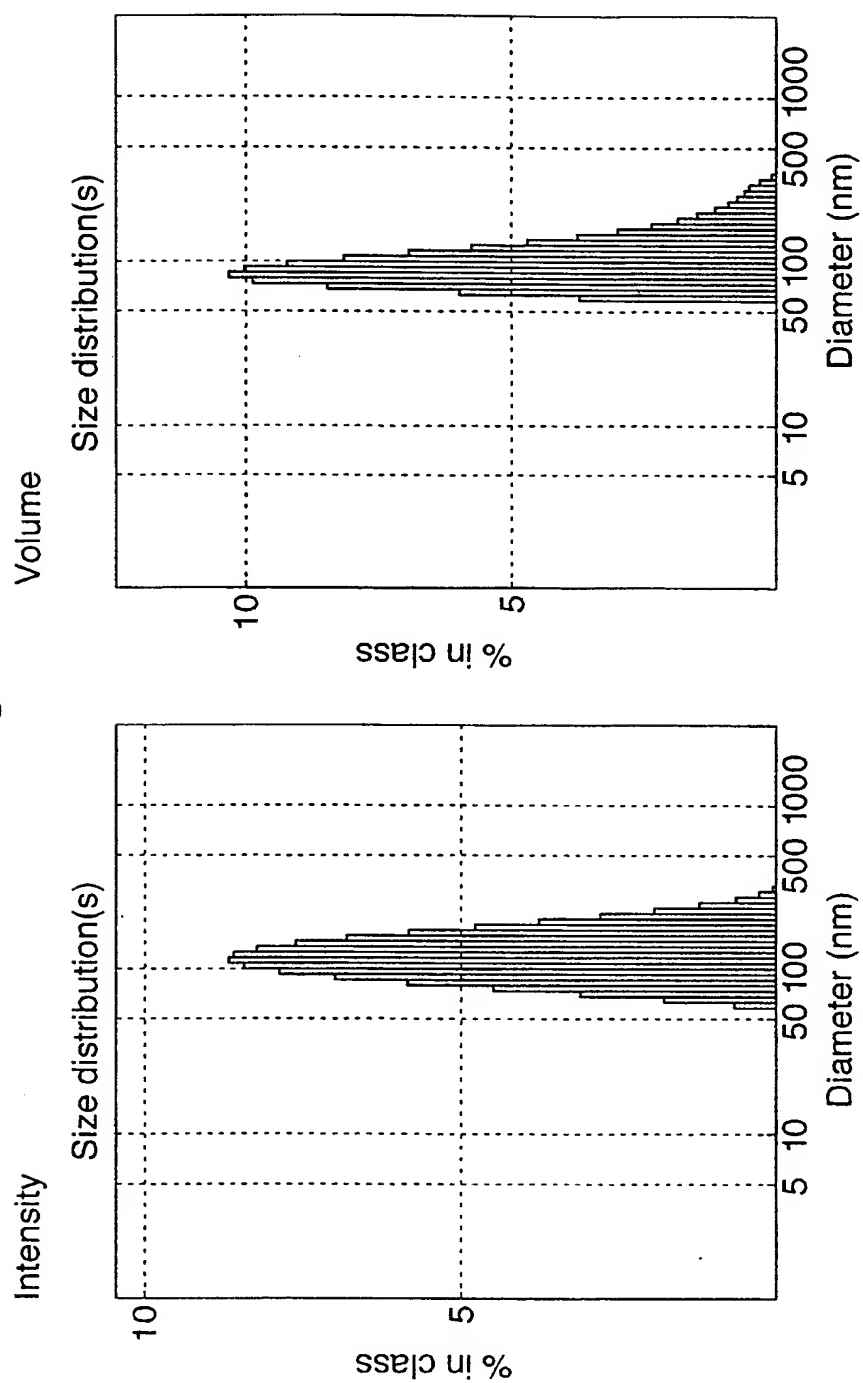
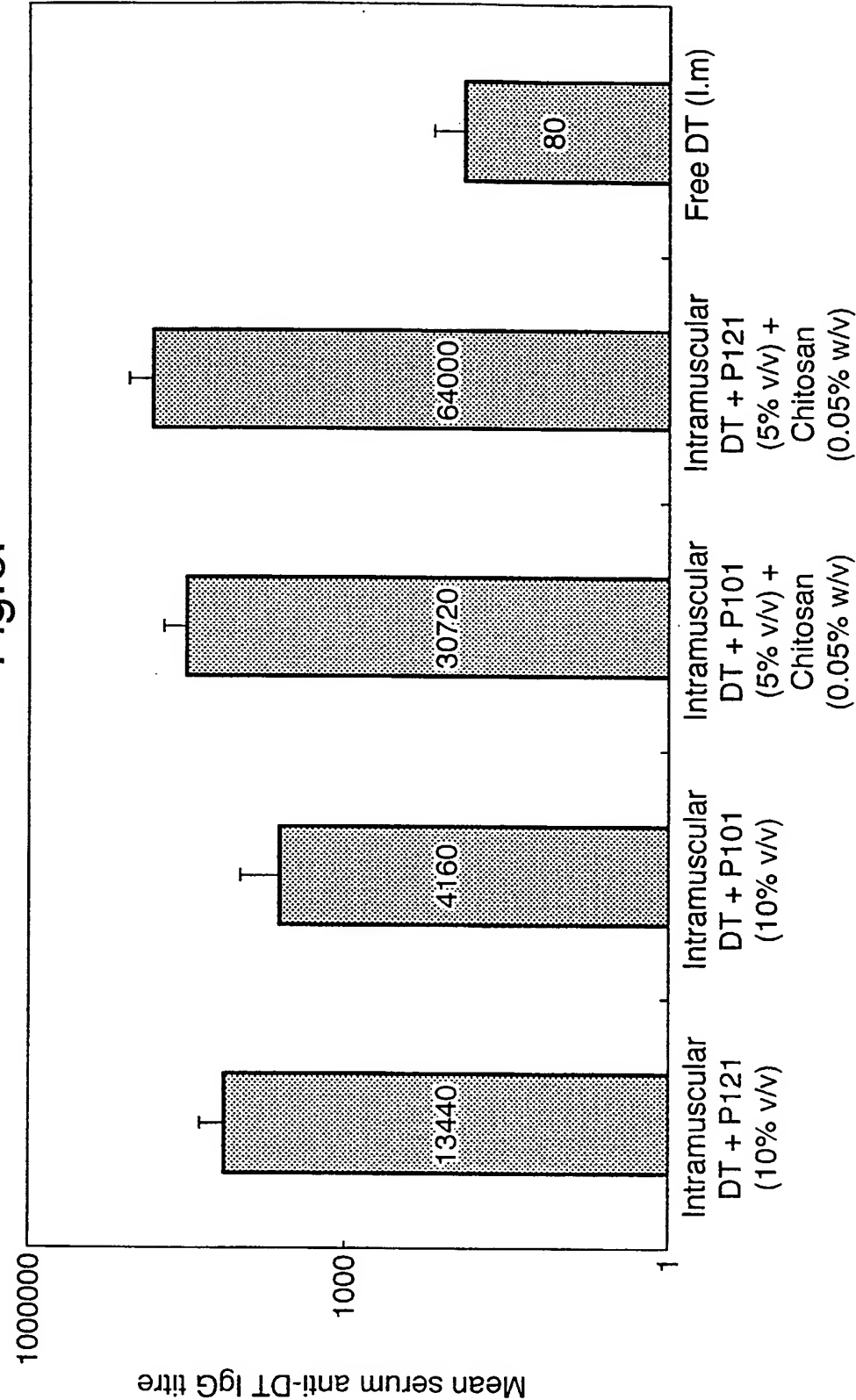


Fig.6.



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(54) Title: **POLYCATIONIC CARBOHYDRATES AS IMMUNOSTIMULANTS IN VACCINES**

(57) Abstract: A polycationic carbohydrate such as chitosan, or a pharmaceutically acceptable derivative thereof, are used as immunostimulants. Vaccine compositions containing these polycationic carbohydrates, in particular in particles such as microparticles or liposomes are also described and claimed. Methods of treatment and the use of the polycationic carbohydrates as immunostimulants in the production of vaccines are further aspects described and claimed.



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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

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A61P31/04

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, PAJ, WPI Data, MEDLINE, EMBASE, LIFESCIENCES, AIDSLINE,
CANCERLIT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 20576 A (DANBIOSYST) 12 June 1997 (1997-06-12) page 27 -page 30 ---	1-9, 23-29
X	WO 96 10421 A (MEDEVA HOLDINGS) 11 April 1996 (1996-04-11) page 17 -page 19 ---	1-9, 23-29
A	US 5 585 106 A (GRISTINA A.G. ET AL.) 17 December 1996 (1996-12-17) the whole document --- -/--	1-29



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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A	<p>KOTZE AWIE F ET AL: "Enhancement of paracellular drug transport with highly quaternized N-trimethyl chitosan chloride in neutral environments: In vitro evaluation in intestinal epithelial cells (Caco-2)."</p> <p>JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 88, no. 2, February 1999 (1999-02), pages 253-257, XP000792263</p> <p>ISSN: 0022-3549</p> <p>the whole document</p> <p>-----</p>	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9720576 A	12-06-1997	AU 705452 B	20-05-1999
		AU 1102597 A	27-06-1997
		CA 2237529 A	12-06-1997
		EP 0865297 A	23-09-1998
		GB 2322801 A, B	09-09-1998
		JP 2000501412 T	08-02-2000
		NO 982497 A	02-06-1998
WO 9610421 A	11-04-1996	AU 710930 B	30-09-1999
		AU 3526995 A	26-04-1996
		BR 9509190 A	21-10-1997
		CN 1159761 A	17-09-1997
		EP 0784485 A	23-07-1997
		JP 10506405 T	23-06-1998
		US 6048536 A	11-04-2000
US 5585106 A	17-12-1996	US 5591441 A	07-01-1997
		US 5292513 A	08-03-1994
		US 5770234 A	23-06-1998
		CA 2135784 A	25-11-1993
		EP 0671952 A	20-09-1995
		JP 8501062 T	06-02-1996
		WO 9323079 A	25-11-1993